Improving the chromatographic selectivity of β-lactam residues analysis in milk using phenyl-column chemistry prior to detection by tandem mass spectrometry

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Abstract

Analyte isobaric interferences can limit the development of a comprehensive analytical method for the quantitative liquid chromatography-tandem mass spectrometry profiling of an important cohort of veterinary drugs. In this work, a selective chromatographic separation was developed for the analysis of 32 β-lactam antibiotic residues (12 penicillins, 14

cephalosporins, five carbapenems and faropenem) in milk samples. A range of analytical columns with different stationary phases and mobile phases were evaluated for retention and separation of the β-lactam compounds. Results showed that, among the columns tested, only phenyl-hexyl could adequately separate ampicillin from cephalexin and amoxicillin from cefadroxil, which had shown isobaric interferences on a number of stationary phases. Chromatography was performed using a water/acetonitrile binary gradient with formic acid and ammonium acetate. The β-lactam residues were extracted from the milk samples using a water:acetonitrile solution and purified by C_{18} dispersive solid-phase extraction (d-SPE) clean-up, followed by concentration under nitrogen and ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) determination. Analytes were monitored in positive electrospray ionization mode (ESI(+)). Possible interfering matrix effects were overcome by using 13 internal standards. The method was fully validated according to 2002/657/EC guidelines, showing satisfactory performance characteristics. Under within-laboratory reproducibility conditions, trueness and precision ranged from 91% to 130% and from 1.4% to 38.6%, respectively. Decision limits (CCα) were in the range 2.1-133 μg kg^{-1}. Limits of detection (LODs) and quantitation (LOQs) ranged between 0.0090 and 1.5 μg kg^{-1} and from 0.030 to 5.0 μg kg^{-1}, respectively.

1. Introduction

β-Lactam antibiotics are widely used in lactating dairy cattle for the treatment of infections. Residues of antibiotics may be found in milk following improper administration or if withdrawal periods are not respected, and may result in adverse effects on human health and development of antibiotic-resistant bacteria. Moreover, these residues can interfere with

cheesemaking process by inhibiting the growth of starter cultures, causing technological problems in dairy industry [1]. A review comparing different researches on antibiotic residues in milk in published literatures found the β-lactams to be the most detected antibiotics, followed by tetracyclines, fluoroquinolones, sulfonamides and aminoglycosides [2].

Penicillins and cephalosporins are the most important β-lactams used in both human and veterinary medicine, while carbapenems play a fundamental role and are approved in human medicine only. In order to minimise exposure and protect public health, Maximum Residue Limits (MRLs) have been established by Commission Regulation 2010/37/EU for eight cephalosporins and seven penicillins in milk (Table 1), some of which are as low as 4 μg kg^{-1} [3]. As a consequence, analytical detection methods are required to be very sensitive. These methods should include a number of metabolites, as Commission Regulation 2010/37/EU specifies that the marker residue for cephapirin should be the sum of the parent drug and its main metabolite, namely desacetyl cephapirin (DAC), while results for ceftiofur should be the sum of all residues retaining the β-lactam structure, expressed as desfuroylceftiofur.

The majority of β-lactam analysis of milk samples is carried out at dairy processing plants by using low cost inhibition assays that allow rapid analysis of large numbers of samples, while more sensitive laboratory-based methods have been developed using microbiological, biosensor and immunochemical techniques [4]. However, the need for confirmation and accurate quantitation has led to the application of chromatographic-based methods coupled to detectors such as UV, fluorescence or mass spectrometry. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) are often used because of the requirement to analyse a wide range of compounds simultaneously in a relatively short analytical run time. Mass spectrometry has also become the technique of choice because of its selectivity, which is particularly important in the case of β-lactams due to the similarity in their molecular

74 structures [5]. However, chromatographic selectivity is also required when co-eluting
75 compounds with similar masses show very similar fragmentation. Reversed-phase
76 chromatography on C_{18} columns is the most common analytical tool in the analysis of β-
77 lactams [6, 7], although retention of polar compounds and selectivity of structurally similar
78 molecules can be a challenge and thus require alternative phases such as phenyl and biphenyl,
79 mixed-mode or hydrophilic interaction liquid chromatography (HILIC) [8, 9].
80 The aim of this work was to develop a sensitive and accurate UHPLC-MS/MS method to
81 measure 32 β-lactam residues in milk. The method consists of all the regulated β-lactams [3],
82 including two main drugs, namely cephapirin and ceftiofur, as well as their major
83 metabolites, and a number of unique non-regulated compounds at target levels (TLs) that
84 were established based on the sensitivity of the method (Table 1). Among these non-MRL
85 compounds, the carbapenems, faropenem, and a number of penicillins and cephalosporins
86 were selected because of their importance in human medicine. The analysis of these drugs in
87 foodstuffs can, indeed, allow to determine whether an illegal or off-label use was employed,
88 therefore preventing the possibility of veterinary residues reaching consumers through the
89 food chain. This is extremely important due to the rising resistance to cephalosporins and the
90 emergence of carbapenem resistance [10]. As a consequence, analytical methods able to
91 determine as many β-lactams as possible, at low levels, are of importance, and the outlined
92 study focused on a large number of drugs that are normally not included in the vast majority
93 of published papers reported in the literature. Another particularly important objective was
94 the evaluation of novel chromatographic stationary phases to improve the separation of
95 structurally similar β-lactams, namely amoxicillin from cefadroxil and ampicillin from
96 cephalexin. These compounds had shown isobaric interferences following application of
97 conventional columns such as C_{18} [11], which could not give satisfactory separation and

could lead to ambiguities in quantitation or confirmation of the residues when applied to the analysis of milk.

2. Materials and Methods

2.1. Chemicals, materials and apparatus

Acetonitrile (MeCN) and methanol (MeOH) were HPLC grade and supplied by Romil Ltd (Cambridge, UK). Ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore water purification system (Cork, Ireland). Dimethyl sulfoxide (DMSO), formic acid (HCOOH) 98-100%, ammonium formate and ammonium acetate were supplied by Sigma-Aldrich (Dublin, Ireland). Glacial acetic acid (CH_3COOH) 100% was purchased from Merck (Darmstadt, Germany).

Polypropylene tubes (15 mL and 50 mL) with screw caps were obtained from Sarstedt Ltd (Wexford, Ireland). QuEChERS 500 mg C_{18} (endcapped) 50 mL centrifuge tubes were purchased from United Chemical Technologies Ltd (Wexford, Ireland). Syringeless mini-uniprep PTFE filter devices were sourced from Whatman plc (Maidstone, UK). A ME36S microbalance and an A200S digital electronic analytical balance (both from Sartorius, Dublin, Ireland) were used for standard preparation. A Talboys Advanced Multi Tube Vortexer (Troemner, NJ, USA), a Rotanta 460R refrigerated centrifuge (Hettich, Kirchlengern, Germany) and a TurboVap LV evaporator (Biotage, Uppsala, Sweeden) were employed for sample preparation.

Ampicillin trihydrate, cloxacillin sodium salt monohydrate, dicloxacillin sodium salt hydrate, mecillinam, methicillin sodium salt, nafcillin sodium salt monohydrate, oxacillin sodium salt monohydrate, penicillin V potassium salt, cefalonium hydrate, ceftiofur, cephapirin sodium,

biapenem, doripenem monohydrate, meropenem trihydrate and faropenem sodium hydrate were obtained from Sigma-Aldrich. Ceftiofur metabolites, namely desfuroylceftiofur cysteine disulfide (DCCD) and desfuroylceftiofur dimer (DCD) were kindly provided by Zoetis (Kalamazoo, MI, USA). Amoxicillin trihydrate, penicillin G potassium salt, piperacillin, ticarcillin monosodium, cefadroxil hydrate, cefazolin sodium salt, cefoperazone sodium salt, cefotaxime sodium salt, cefquinome sulphate, cefuroxime sodium salt, cephalexin monohydrate and imipenem were purchased from LGC Standards (Teddington, Middlesex, UK). Cefacetrile, desacetyl cephapirin (DAC) sodium salt, ertapenem disodium 90%, amoxicillin-d_4, ampicillin-d_5, benzyl penicillanate-d_7 potassium salt (penicillin G-d_7), nafcillin-d_5 sodium salt, penicillin V-d_5, cefadroxil-d_4 (major), cefazolin-$^{13}C_2{}^{15}N$ sodium salt, cefquinome-d_7 hydroiodide (90%), cephalexin-d_5 hydrate, DAC-d_6 sodium salt (major), DCCD-d_3, meropenem-d_6 >90% and piperacillin-d_5 were supplied by Toronto Research Chemicals (Toronto, ON, Canada).

2.2. Preparation of standards

Individual stock solutions were prepared as described by Di Rocco et al. [11] by dissolving the appropriate amount of standards in H_2O:MeCN (75:25, *v/v*), H_2O:MeCN (50:50, *v/v*) or DMSO, depending on their solubility. In addition, ceftiofur was dissolved in DMSO at a concentration of 0.5 mg mL^{-1}, while the internal standards cefquinome-d_7, meropenem-d_6 and piperacillin-d_5 were dissolved in DMSO at a concentration of 1 mg mL^{-1}. Stock solutions were found to be stable for at least 12 months when stored in 2.5 mL aliquots in 15 mL polypropylene tubes at -80°C, with the exception of DCD and mecillinam, which were stable for four and six months only, respectively.

As described in Online Resources 1 and 2 (supplementary materials), the β-lactam stock solutions were organised in three different groups, and an appropriate volume of each stock

148 was diluted in water in order to obtain three separate intermediate mixed solutions (IS1-3)
149 containing the β-lactams at concentrations ranging from 4.0 µg mL^{-1} to 50 µg mL^{-1}.
150 Subsequently, the intermediate solutions were combined and further diluted in water to obtain
151 eight working standard solutions (WSS1-8). An appropriate volume of each internal standard
152 stock solution was diluted directly in water or through intermediates (IS4-7) to obtain a
153 mixed internal standard solution at concentrations ranging from 0.080 µg mL^{-1} to 2.0 µg mL^{-1}
154 (Online Resources 3 and 4, supplementary materials). Working solutions were stored at -
155 80°C and prepared monthly.
156
157 *2.3. Milk samples*
158 Raw bovine milk samples were obtained from different Irish farms and stored at -80°C prior
159 to analysis. The samples were subsequently tested by applying the proposed method, found to
160 be drug-free and used as negative controls.
161
162 *2.4. Preparation of extracted matrix calibrants and recovery controls*
163 Extracted milk matrix calibrants were prepared by fortifying negative samples (2 g ± 0.01 g)
164 prior to extraction with 100 µL of each working standard solution, in order to give eight point
165 calibration curves in the ranges reported in Table 1.
166 Recovery controls were prepared by spiking four negative milk samples post-extraction, two
167 with 100 µL of WSS2 and two with 100 µL of WSS7, in order to monitor for loss of analytes
168 during sample preparation and evaluate the extraction efficiency for each analyte at a low and
169 a high concentration over the calibration range of the method.
170
171 *2.5. Sample preparation*

Samples (2 g ± 0.01 g) were weighed into a polypropylene centrifuge tube (50 mL). A 100 µL volume of the mixed internal standard solution was added to all calibrants, recovery controls and test samples. Subsequently, the samples were gently shaken for few seconds and allowed to stand for 15 min. A volume of water (0.9 mL) was added to all calibrants, while 1 mL of water was added to the recovery controls and test samples. A volume of MeCN (7 mL) was added to all tubes, which were then vortexed for 1 min using a multi-tube vortexer. The samples were centrifuged for 15 min at 2842×*g* (4°C), and the supernatant was subsequently decanted into a 50 mL d-SPE polypropylene tube containing 500 mg of endcapped C_{18} sorbent. The tubes were vortexed (40 s) and centrifuged for 15 min at 2842×*g* (4°C). After centrifugation, the entire supernatant was transferred into a 15 mL polypropylene tube using a mechanical pipette, and evaporated under nitrogen on a TurboVap at 40°C to a final aqueous volume of <1 mL (which took approximately 70 min). The volume was then made up to 1 mL with water. The extracts were vortexed for 10 s and centrifuged for 15 min at 2842×*g* (4°C). A 400 µL aliquot of the final extract was transferred into syringeless mini-uniprep PTFE devices, filtered and injected into the UHPLC-MS/MS system.

2.6. Optimisation of MS/MS conditions

In order to identify precursor and product ions, tuning was performed on an Agilent 6470 triple quadrupole mass spectrometer by injecting 1 µL of 1 µg mL^{-1} aqueous solutions of standards and internal standards, with mobile phase A:B (50:50, *v/v*) at a flow rate of 0.2 mL min^{-1}. The selected transitions are presented in Table 2. An optimisation of the source parameters was subsequently performed to maximize the sensitivity achieved for each compound.

2.7. UHPLC-MS/MS conditions

Analysis was performed using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies Ltd, Cork, Ireland) equipped with jet stream electrospray ionisation (AJS ESI) probe, and controlled by an Agilent MassHunter software. Chromatography was achieved on an Agilent Zorbax Eclipse Plus Phenyl-Hexyl Rapid Resolution HD analytical column (3.0×100 mm, 1.8 µm particle size) fitted with an in-line filter with a 0.2 µm pore size, using a binary gradient comprising of 0.01% HCOOH and 0.2 mM ammonium acetate in H_2O (mobile phase A) and 0.01% HCOOH in MeCN (mobile phase B). The flow rate was set at 0.4 mL min^{-1}. The total run time was 12 min, and the gradient profile as follows: (1) 0 – 0.5 min, 100% A; (2) 0.5 – 2.5 min, linear decrease to 80% A; (3) 2.5 – 7.5 min, linear decrease to 20% A; (4) 7.5 – 7.6 min, linear decrease to 0% A; (5) 7.6 – 9.0 min, hold at 0% A to wash the column with organic mobile phase B; (6) 9.0 – 9.1 min, linear increase to 100% A, which was held for 2.9 min to allow re-equilibration of the column. The column compartment was maintained at 30°C, while the multisampler temperature was set at 7°C. The injection volume was 10 µL. The autosampler was rinsed after each injection using a solution of H_2O:MeCN (50:50, *v/v*). A divert valve was used to reduce source contamination from undesired matrix components (solvent delay: [a] 0 – 2.56 min; [b] 7.77 – 12 min).

The mass spectrometer was operated in positive ESI mode (ESI(+)). The optimised source operating parameters were: gas temperature, 150°C; sheath gas temperature, 400°C; gas flow, 9 L min^{-1}; sheath gas flow, 11 L min^{-1}; nebulizer, 45 psi; capillary, 5250 V; nozzle voltage, 1000 V. The cycle time was set at 0.5 s.

2.8. Method validation

The method was validated as described by Di Rocco et al. [11], following 2002/657/EC guidelines [12]. Trueness, within-laboratory repeatability (WLr) and within-laboratory

reproducibility (WLR) were assessed for the MRL-substances and the metabolites at 0.5, 1 and 1.5 times the MRLs set by current legislation [3]. For the non MRL-substances, TLs were established based on the sensitivity achieved by the method for each compound, and validation was performed at 0.5, 1 and 1.5 times the TLs. The WLr study was performed on three different days by the same analyst, using the same negative sample and by fortifying eight replicates for each validation level. The WLR study was performed on three separate days by three different analysts by fortifying 24 different control milk samples (eight samples for each validation level). These samples were obtained from bulk tanks of 24 different Irish farms.

2.9. Data analysis

Linear regression of the calibration data was performed with a weighing factor of $1/x^2$. Thirteen internal standards were used for quantitation purposes. Each labelled compound was employed for its corresponding analyte. In addition, results for cefacetrile, ertapenem and mecillinam were corrected using cefadroxil-d_4; for cefalonium, cefotaxime, cefuroxime and faropenem using cefquinome-d_7; for cloxacillin and dicloxacillin using nafcillin-d_5; for doripenem using amoxicillin-d_4. Among the available labelled compounds, no suitable internal standards could be identified for biapenem, imipenem, cefoperazone, ceftiofur, cephapirin, DCD, methicillin, oxacillin and ticarcillin during matrix effect investigations, therefore no internal standards were used in their quantitation (refer to *3.2.2* for further details).

3. Results and discussion

246 *3.1. Method development*

247

248 *3.1.1. Chromatographic separation*

249 A chromatographic separation based on a method previously published by our group for the
250 analysis of β-lactam residues in bovine muscle was initially evaluated [11]. The application
251 of this method identified isobaric interferences between two pairs of analytes, namely
252 amoxicillin-cefadroxil and ampicillin-cephalexin, most likely due to the similar masses of
253 these compounds and their isotopes (Figure 1). Additionally, despite the attempt to select
254 unique transitions for each analyte, the same product ions were obtained in CID experiments
255 (Figure 1 and Figure 2), most of which have been characterised in the literature [13]. This led
256 to interference from cefadroxil and cephalexin in the analysis of amoxicillin and ampicillin,
257 respectively, resulting in inaccuracy for quantitation purpose or ambiguity in analyte
258 confirmation. This did not represent a problem in the previously developed method for β-
259 lactam analysis in bovine muscle [11], as the same, or very similar, calibration ranges could
260 be established for the two pairs of analytes, resulting in negligible interference. However, in
261 this work, different calibration ranges had to be established to allow the analysis of milk at
262 very different MRL levels (range 4-125 μg kg^{-1}, Table 1), and to ensure adequate MS
263 sensitivity for cefadroxil. If these compounds are not chromatographically resolved in multi-
264 residue analysis, it will result in underestimation of amoxicillin and ampicillin concentrations
265 in test samples, which could potentially lead to false negative results.
266 Three potential alternative approaches could be considered: (1) the validation of a low-level
267 screening method for cephalexin and cefadroxil, and a separate high level quantitative
268 method to be applied in case of positive samples found; (2) a separate validation for the two
269 analytes of each pair; (3) the use of high-resolution mass spectrometry (HRMS), which would
270 offer high mass accuracy, allowing to easily distinguish those analytes. However, approaches

(1) and (2) would be more expensive and time-consuming when applying the method for routine analysis, while approach (3) would imply a higher cost compared to low resolution MS, and it could also be not always available in routine laboratories. Therefore, a range of different chromatographic conditions were evaluated in order to achieve separation of each pair of cross-interfering analytes. Additional objectives of this research were the improvement of column retention of early eluting polar analytes (namely imipenem and biapenem), and the reduction of the chromatographic cycle to <18 min total run time, mostly due to the long re-equilibration time.

A range of different mobile phase additives and concentrations (0.01-0.1% formic acid and acetic acid; 0.2-50 mM ammonium formate and ammonium acetate) were initially tested on the CSH C_{18} analytical column employed for the meat method [11]. However, it became quickly apparent that conventional stationary phases lacked the selectivity to separate both co-eluting pairs when trying to maintain a relatively short total run time. In fact, our previous research had already shown that other columns such as BEH C_{18} and HSS T3 could separate amoxicillin from cefadroxil, but not ampicillin from cephalexin [11]. Therefore, the following alternative columns were assessed based on solvent and additive compatibilities and physico-chemical properties of the compounds:

- Phenomenex Kinetex PFP 100 × 2.1 mm, 1.7 μm;
- Fortis SpeedCore PFP 100 × 2.1 mm, 2.6 μm;
- UCT Selectra PFPP 10 cm × 2.1 mm, 3 μm;
- Sielc Obelisc R 2.1 × 100 mm, 5 μm;
- Imtakt Scherzo SS-C_{18} 100 × 2 mm, 3 μm;
- Imtakt Scherzo SM-C_{18} 100 × 2 mm, 3 μm;
- Agilent Zorbax Eclipse Plus Phenyl-Hexyl RRHD 3.0 × 100 mm, 1.8 μm;
- Fortis SpeedCore DiPhenyl 100 × 2.1 mm, 2.6 μm.

Separation was considered as successfully achieved if peak resolution (R_s) was ≥1.5 [14].

Fluorinated stationary phases, especially pentafluorophenyl (PFP) moieties, are often used as alternatives to common C_{18} and C_8 phases to promote dipole, π-π and strong ion-exchange interactions. In this study, these columns did not show satisfactory separation of the co-eluting analytes, with the exception of SpeedCore PFP, which could separate amoxicillin and cefadroxil, but not ampicillin and cephalexin. Multi-mode columns were also evaluated, including Obelisc R, Scherzo SS-C_{18} and Scherzo SM-C_{18}. These columns allow the separation of analytes using different chromatographic separation processes, namely partition-based retention and ion exchange. The Obelisc R was found to provide satisfactory separation of ampicillin and cephalexin, but could not resolve amoxicillin from cefadroxil. In addition, peak shape was found to be unsatisfactory. In contrast, the Scherzo columns separated amoxicillin and cefadroxil, but did not resolve the other problematic pair, and poor sensitivity was also obtained overall for all analytes due to the need to include 50 mM ammonium formate in the mobile phase.

Considering the chemical structure of the problematic analytes, the phenyl-hexyl and diphenyl columns were evaluated due to their ability for improving the selectivity of aromatic compounds [9] and the retention of highly polar drugs [8]. In this study, these were the only two columns that achieved satisfactory separation for both pairs of analytes (Figure 2). However, the phenyl-hexyl column combined with a water/acetonitrile mobile phase seemed to be the most reasonable choice because it also provided better retention of the more polar analytes (which started to be eluted after 3 min compared to 2.3 min on the C_{18} column), gave better overall sensitivity, and allowed reduction of the total run time to 12 min.

Subsequently, three different batches of the phenyl-hexyl column were tested to verify reproducibility of the retention times. The maximum standard deviation (SD, n=3) was ±0.05 min, which was deemed to be robust for routine application.

321

322 *3.1.2. Storage of milk samples*

323 A number of stability studies have been reported in the literature for β-lactam antibiotics in
324 milk samples. Hou *et al.* [15] investigated the stability of 10 cephalosporins and desacetyl
325 cephapirin in fortified bovine milk samples stored at -20ºC over a period of 14 days.
326 Although no degradation was observed after seven days, significant loss was reported for
327 cephalexin, ceftiofur and cefacetrile on day 14. Riediker *et al.* [16] investigated the stability
328 of five penicillins, namely amoxicillin, ampicillin, cloxacillin, oxacillin, and penicillin G, in
329 fortified milk samples at different storage temperatures (+4, -20 and -76ºC) over a period of
330 28 days. The authors observed that all analytes were stable for a maximum of three days if
331 samples were stored at +4ºC. When stored at -20ºC, amoxicillin and ampicillin were the only
332 two analytes stable throughout the chosen storage period, while the other compounds
333 underwent between 10-20% degradation after three days. No degradation was reported for
334 samples stored at -80ºC throughout the entire storage period. However, the authors employed
335 pasteurized milk, rather than raw milk. A number of studies have also been reported for β-
336 lactam stock solutions, highlighting the challenge in the analysis of these compounds and the
337 importance of storage temperatures below -70ºC [17-19, 11]. As a consequence, it was
338 decided to store all the milk samples used for the proposed study at -80ºC to avoid potential
339 degradation of the residues, if present.

340 *3.1.3. Sample preparation*

341 In order to isolate the β-lactam residues and precipitate milk proteins, two different extraction
342 solutions (8 mL of MeCN and 8 mL of MeCN:H_2O (7:1, *v/v*)) were investigated, and absolute
343 recoveries evaluated as described by Di Rocco et al. [11]. The use of MeCN only gave lower
344 recoveries for the more polar penicillins and cephalosporins, the carbapenems, faropenem
345 and the ceftiofur-related metabolites, providing overall results ranging from 11% (ertapenem)

346 to 92% (methicillin). The polarity of the solution was subsequently increased with the
347 addition of 1 mL of water, which significantly improved the efficiency of the extraction and
348 provided satisfactory absolute recoveries ranging from 60% (amoxicillin and ertapenem) to
349 118% (methicillin) (Figure 3).
350 Solvent evaporation and analyte concentration under nitrogen were required to achieve
351 satisfactory sensitivity for the regulated compounds with low MRLs. The importance of
352 temperature control during sample preparation and UHPLC-MS/MS analysis for β-lactams
353 has already been deeply investigated [11]. Therefore, it was decided to keep the solvent
354 evaporation temperature at 40ºC, while dynamically increasing the nitrogen gas pressure
355 from 10 to 20 psi. This allowed the evaporation of MeCN in approximately 70 min. On the
356 other hand, the C_{18} d-SPE clean-up and the use of internal standards avoided adverse matrix
357 effects that could be caused by a significant amount of matrix components in the final extract.
358
359 *3.2. Method validation*
360
361 *3.2.1. Confirmatory criteria, selectivity, linearity, LODs and LOQs*
362 As outlined in the 2002/657/EC guidelines, three identification points are required by a
363 confirmatory method for group B substances. A minimum of four identification points were
364 obtained for all analytes in the proposed work. In addition, retention time and ion ratio
365 deviations were all within the maximum permitted tolerances [12]. The selectivity study
366 showed no interferences when injecting all standards and internal standards individually, and
367 no matrix undesired peaks when injecting 25 blank milk samples obtained from different
368 sources. Satisfactory linearity was achieved over the calibration range of the method, as $R^2 \geq$
369 0.98 were obtained for all analytes, with a five-point calibration curve (excluding the origin)

achieved for all compounds and individual residuals in the ± 20% range of deviation tolerance from the calibration curve.

Although the assessment of the LODs and LOQs is not required by 2002/657/EC guidelines, these parameters were estimated based on the lowest signal-to-noise (S/N) ratio obtained for the first calibration point among the six validation runs (Table 3). The LOD and LOQ values were set at those concentration levels for which the S/N ratio for the qualifier ion would be at least 3 and 10, respectively. The estimation was made based on the S/N ratio of the least abundant ion in order to take into account the variability that could be observed between different milk samples, and report a more realistic estimation of the LOD and LOQ values. The estimated LODs were in the range 0.0090-1.5 μg kg^{-1}, while LOQs ranged from 0.030 to 5.0 μg kg^{-1} (Table 3).

3.2.2. Matrix effects

As reported in Table 3, the matrix effect study showed matrix enhancement from 0.25% (ertapenem) to 65% (faropenem) for all compounds, except for amoxicillin, mecillinam, cefacetrile, cefadroxil, DCD, biapenem and doripenem, for which matrix suppression was observed (range 3.6-29.4%). It is particularly important to evaluate the effect of matrix variability for the quantitation of those analytes that show matrix enhancement, due to the fact that a positive sample could lead to a false non-compliant result. Therefore, 25 milk samples from different origin were fortified post-extraction at the MRLs/TLs and compared to a mixture of standards in solvent at the same concentrations. When available, the corresponding labelled compound was used as an internal standard in the quantitation of the analyte. The effect of each internal standard on the quantitation of the other β-lactams was also evaluated so that a suitable internal standard could be identified. Variability between the different samples was significantly reduced when using a labelled compound, with relative

standard deviation (RSD%) values <16.4% except for faropenem (38.9%), for which the use of cefquinome-d_7 did not show significant improvement. However, quantitation against the internal standard was applied to compensate for any loss during sample preparation or instrument variability. Although no suitable internal standards could be identified for methicillin, oxacillin, ticarcillin, cephapirin, cefoperazone, ceftiofur, DCD, biapenem and imipenem, variability was ≤ 22.3%, with the only exceptions of ceftiofur (28.2%) and DCD (27.3%).

3.2.3. Trueness, precision, CCα and CCβ

As reported in Table 4, the trueness under WLr conditions was satisfactory and within the range 91-110% for most of the compounds, with the only exceptions of oxacillin and DCD at 1.5 MRL level, for which the trueness was slightly above the maximum acceptable values (115% and 111%, respectively) [12]. The trueness was also satisfactory for the majority of substances under WLR conditions (range of 91-110%). The exceptions were at 1.5 MRL/TL for ticarcillin (119%), cephapirin (112%), cefoperazone (121%) and DCD (130%), for which no suitable internal standards could be identified during the method development work. Precision for most analytes satisfied the 2002/657/EC requirements, and was ≤ 23% for the majority of analytes. Under WLr conditions, CVs% were in the range 0.86-9.7%, except for DCD at 0.5 MRL, for which CV was 17.7%. Under WLR studies, CVs ranged from 1.4% to 21.8% for most of the compounds. Precision was still acceptable for faropenem at 0.5 TL and TL (24.6% and 25%, respectively), and for cefotaxime and ticarcillin at TL (25.5% and 29.9%, respectively), as it was found to be lower than the maximum coefficient of variations (CVs%) calculated by the Horwitz equation [12]. The precision of the method was above maximum CVs for DCD (25.3% at 0.5 MRL and 29.4% at MRL), ticarcillin (38.6% at 0.5 TL and 36.0% at 1.5 TL) and cefoperazone at all validation levels (34.7% at 0.5 MRL, 29.3% at

MRL and 35.1% at 1.5 MRL). This issue could be addressed by implementing suitable corresponding labelled internal standards into the method. CCα values ranged from 2.1 µg kg^{-1} (mecillinam) to 133 µg kg^{-1} (cefacetrile), depending on the analyte.

In our previous work [11] and in the literature [20], a rapid conversion of cephapirin to desacetyl cephapirin occurred in fortified muscle samples and kidney homogenate, while this conversion was not observed in milk samples. Therefore, in contrast to the meat method [11], both cephapirin and its metabolite could be included and successfully validated in the proposed work, allowing compliance with current legislation [3].

3.3. Comparison with existing methods

A review of the LC-MS published methods that were specifically developed for the analysis of β-lactam residues in milk showed that most of the sample pretreatments employ SPE on C_{18} or Oasis HLB cartridges [7, 21, 6, 22], a relatively time-consuming and expensive procedure, with significant amounts of solvents required [23]. Baenza et al. [24] applied molecularly imprinted polymers (MIPs) for the determination of cephalosporin residues in bovine milk. However, the method included six cephalosporins only, with mean recoveries in the range 15-100%. . Van Holthoon et al. [25] described a LC-MS/MS method for eight penicillins in milk, with a sample pretreatment that required derivatisation with piperidine followed by SPE. Online SPE, ultrasound-assisted matrix solid phase dispersion (MSPD) combined with QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique, and dispersive liquid-liquid microextraction (DLLME) have also been reported for the analysis of β-lactams in milk [4, 26, 23]. These methods include a limited number of penicillins and cephalosporins, and do not target the main metabolites. An alkaline QuEChERS extraction employing a mixture of anhydrous sodium sulphate (Na_2SO_4) and sodium chloride (NaCl) with mechanical shaking was applied by Bessaire et al. [27] for the analysis of 23 β-lactams,

including desacetyl cephapirin, in foods of animal origin (eggs, raw milk, processed dairy ingredients, baby foods, meat- and fish-based products). This work included a quite wide range of analytes, but it was only applied for screening purposes, as significant losses of the compounds were observed during sample preparation, with absolute recoveries in the range 8-55%, especially for the more polar analytes.

Among the most recent published methods, the majority of authors focused on the inclusion of a wide range of veterinary drugs from different classes. Turnipseed *et al.* [28] developed a quadrupole time-of-flight (Q-TOF) LC-MS method for four different classes of veterinary active compounds, including three penicillins and one cephalosporin, in milk. The samples were extracted using acetonitrile, and clean-up was subsequently performed using 3000Da molecular weight cut-off centrifuge filters. Zhu *et al.* [29] developed a LC-MS/MS method for 88 veterinary drugs from eight different families, including eight penicillins. Samples were ultrasonic extracted, and subsequently purified using TurboFlow online SPE. Castilla-Fernández *et al.* [30] evaluated different sample preparation procedures for the determination of 66 veterinary drugs, including a fast pass-through SPE clean-up on HLB PRiME cartridges. The study focused on more than 14 different drug classes, and included three penicillins and three cephalosporins. Despite the possibility to monitor more drug families simultaneously and the fact that these methods are faster, less time-consuming and cheaper than single-class methods, they included only a limited number of β-lactams.

The proposed study employs a simple and fast dSPE-based sample preparation procedure for the extraction of 32 β-lactam residues from milk matrix, including the carbapenems for which, to the best of our knowledge, there are no other published methods. Absolute recoveries for the 32 compounds ranged from 60 to 118%, demonstrating the efficiency of the extraction procedure. As shown from validation results, the method is also able to

accurately quantify the residues, and matches the recommended performance criteria reported by 2002/657 (EC) guidelines [12].

One of the main objectives of this work was also to develop a highly selective method. Due to the class-specific fragments obtained for many of the β-lactams, it is important to underline the necessity of assessing selectivity in MS/MS analysis, discouraging the common practice of simply choosing the most intense product ions rather than the most distinctive [13]. The choice of unique product ions represents the easiest approach to selectivity issues. However, this is not always achievable for compounds of similar masses and if similar products are generated in CID experiments. Therefore, an efficient chromatography must be developed in order to obtain optimal separation. The majority of methods published for the analysis of β-lactams in milk include penicillins [31, 32, 25, 33] or cephalosporins [24, 15, 26, 1, 34] only. Among the methods that target at least one of the two pairs of problematic analytes, Daeseleire *et al.* [35] obtained satisfactory resolution for ampicillin and cephalexin on a HPLC Alltima C_{18} column using a water/acetonitrile mobile phase with formic acid. Although very good separation was achieved for the two compounds (1.76 min of difference), a 150 mm chromatographic column and a longer gradient had to be employed. As a consequence, the total run time for the separation of the 11 analytes included in the method had to be set to 17 min, considering that a final hold of 7.2 min at 100% mobile phase A was required for the re-equilibration of the system. Similarly, Becker *et al.* [20] employed a water/methanol mobile phase with formic acid on a phenylether column for the separation of 15 penicillins and cephalosporins, including ampicillin and cephalexin. In their method, a 250 mm HPLC column was used, with a total run time of 52 min, including a binary gradient extended over a 22 min time, a 15 min hold at 90% mobile phase B for washing the column, and a final hold at 100% mobile A for system re-equilibration. For the majority of published methods, very similar retention times were obtained for the two problematic pairs of analytes

494 on C_{18} stationary phases [18, 36, 37]. However, the selectivity/specificity studies aimed to
495 identify the presence of undesired peaks from the matrix by analysing blank samples, but did
496 not investigate the possible interference between the different standards. The research
497 proposed in this work underwent a detailed study of possible interferences between all
498 analytes, which is necessary for multi-residue methods in the presence of compounds with
499 similar physico-chemical properties and behaviour, and addressed the issue by developing an
500 efficient chromatographic separation in order to obtain satisfactory resolution for all the
501 drugs.
502
503
504 **Conclusions**
505 The development of a sensitive UHPLC-MS/MS method for the simultaneous analysis of 32
506 β-lactam antibiotic residues in milk was presented. The method included a wide range of
507 unique compounds which are normally not analysed in routine laboratories. Additionally, to
508 the best of our knowledge, this is the first time that a method for carbapenems and faropenem
509 in milk samples is reported. Our research also showed the importance of assessing selectivity
510 when validating a multi-residue method that includes compounds with similar masses and
511 structures. Isobaric interferences were observed between amoxicillin and cefadroxil and
512 between ampicillin and cephalexin, most likely due to naturally occurring isotopes. The
513 method presented in this work addressed this problem by achieving satisfactory
514 chromatographic selectivity through the use of a novel phenyl-hexyl stationary phase, which
515 was demonstrated to be a valid alternative to C_{18} analytical columns to obtain the efficient
516 separation of these β-lactams. The sample preparation procedure involved a simple and fast
517 d-SPE clean-up step, and allowed a single analyst the extraction of 60-70 milk samples per
518 day. The results of the validation study showed a high degree of accuracy in the quantitation

of the majority of analytes included. In routine conditions, this method represents an easy and valid approach for the determination of β-lactam residues in bovine milk samples.

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Declarations

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Table captions

Table 1 Maximum Residue Limits (MRLs)/Target Levels (TLs) for β-lactam antibiotics in milk and calibration ranges of the proposed method

Table 2 UHPLC-MS/MS conditions for β-lactams in milk

Table 3 Calibration ranges, LODs, LOQs and matrix effect data. Positive values indicate ion suppression, while negative values indicate matrix enhancement

Table 4 Trueness (%), precision (%), CCα and CCβ values for β-lactam compounds in raw bovine milk

Figure captions

Fig1(a) MS(/MS) spectra of amoxicillin and cefadroxil. The same product ions (114 *m/z*, 208 *m/z* and 349 *m/z*) were generated in CID experiments from the 366 *m/z* precursor mass

Fig1(b) MS(/MS) spectra of ampicillin and cephalexin. The same product ions (106 *m/z*, 174 *m/z* and 192 *m/z*) were generated in CID experiments from the 350 *m/z* precursor mass

Fig2(a) Injections of individual standards of amoxicillin and cefadroxil at 250 ng mL^{-1}. Chromatograms show the interference from cefadroxil in the analysis of amoxicillin on the CSH C_{18} column (A and B), while analytes are resolved on the phenyl-hexyl column (C and D)

Fig2(b) Injections of individual standards of ampicillin and cephalexin at 250 ng mL^{-1}. Chromatograms show the interference from cephalexin in the analysis of ampicillin on the CSH C_{18} column (A and B), while analytes are resolved on the phenyl-hexyl column (C and D)

Fig3 Absolute recoveries (efficiency of extraction) and standard deviations (shown by error bars, n=3) obtained for β-lactam compounds when using 8 mL of MeCN and 8 mL of MeCN:H_2O (7/1, *v/v*) as extraction solutions. Results for cephapirin and ceftiofur are not shown, as these analytes were added at a further stage of the sample preparation development process

Online Resources captions

Online Resource 1 Flow diagram showing the preparation of the working standard solutions

Online Resource 2 Concentrations of the β-lactams in the three intermediate mixed solutions and range of concentrations in the final working standard solutions

Online Resource 3 Flow diagram showing the preparation of the mixed internal standard solution

Online Resource 4 Concentrations of the β-lactam internal standards in the intermediate and final mixed internal standard solutions

Table 1

Analyte	Animal species (37/2010/EU)	MRL/TL (µg kg^{-1})	Calibration range (µg kg^{-1})
Amoxicillin	All food producing species	4[a]	1-8
Ampicillin	All food producing species	4[a]	1-8
Cloxacillin	All food producing species	30[a]	7.5-60
Dicloxacillin	All food producing species	30[a]	7.5-60
Mecillinam	Bovine	2[b]	0.5-4
Methicillin	-	2[b]	0.5-4
Nafcillin	All ruminants	30[a]	7.5-60
Oxacillin	All food producing species	30[a]	7.5-60
Penicillin G	All mammalian food producing species	4[a]	1-8
Penicillin V	-	4[b]	1-8
Piperacillin	-	4[b]	1-8
Ticarcillin	-	10[b]	2.5-20
Cefacetrile	Bovine	125[a]	31.25-250
Cefadroxil	-	20[b]	5-40
Cephalexin	Bovine	100[a]	25-200
Cephapirin	Bovine	60[a]	15-120
Cefalonium	Bovine	20[a]	5-40
Cefazolin	Bovine, ovine, caprine	50[a]	12.5-100
Cefoperazone	Bovine	50[a]	12.5-100
Cefotaxime	-	8[b]	2-16
Cefquinome	Bovine	20[a]	5-40
Ceftiofur	All mammalian food producing species	100[a]	25-200
Cefuroxime	-	20[b]	5-40
Desacetyl cephapirin	Bovine	60[a]	15-120
Desfuroylceftiofur cysteine disulfide	All mammalian food producing species	100[a]	25-200
Desfuroylceftiofur dimer	All mammalian food producing species	100[a]	25-200
Biapenem	-	6[b]	1.5-12
Doripenem	-	40[b]	10-80
Ertapenem	-	20[b]	5-40
Imipenem	-	20[b]	5-40
Meropenem	-	10[b]	2.5-20
Faropenem	-	4[b]	1-8

[a] = MRL; [b] = TL.

Table 2

Analyte	Monitored ion	RT (min)	Delta RT (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	FRM (V)	CE (eV)	CAV (V)
Imipenem	$[M+H]^+$	3.05	1	300.1	141.9[a]/123.9	110	33/45	4
Biapenem	$[M+H]^+$	3.11	1	351.1	110.0[a]/265.0	110	17/13	4
Amoxicillin	$[M+H]^+$	3.22	1	366.1	349.0[a]/113.9	110	5/21	4
Amoxicillin-d_4	$[M+H]^+$	3.21	1	370.1	353.0	110	5	4
DAC	$[M+H]^+$	3.29	1	382.1	151.9[a]/111.9	140	33/29	4
DAC-d_6	$[M+H]^+$	3.28	1	388.1	232.0	140	17	4
Doripenem	$[M+H]^+$	3.51	1	421.1	274.0[a]/112.0	140	17/50	4
Cefadroxil	$[M+H]^+$	3.53	1	364.1	208.0[a]/113.9	80	5/17	4
Cefadroxil-d_4	$[M+H]^+$	3.53	1	368.1	212.0	80	5	4
Meropenem	$[M+H]^+$	3.69	1	384.2	141.0[a]/340.1	140	13/9	4
Meropenem-d_6	$[M+H]^+$	3.68	1	390.2	147.2	110	13	4
DCCD	$[M+H]^+$	3.83	1	549.0	182.9[a]/241.0	170	25/21	5
DCCD-d_3	$[M+H]^+$	3.82	1	552.1	244.0	170	21	5
Cephapirin	$[M+H]^+$	3.84	1	424.1	292.0[a]/151.9	140	13/25	4
Ampicillin	$[M+H]^+$	3.94	1	350.1	106.1[a]/192.0	110	21/13	4
Ampicillin-d_5	$[M+H]^+$	3.92	1	355.2	111.0	110	21	4
Cephalexin	$[M+H]^+$	4.13	1	348.1	157.9[a]/174.0	110	5/13	4
Cephalexin-d_5	$[M+H]^+$	4.12	1	353.1	158.0	110	5	4
Ertapenem	$[M+H]^+$	4.22	1	476.2	432.0[a]/233.0	140	5/17	4
Cefacetrile	$[M+NH_4]^+$	4.27	1	357.1	280.0[a]/251.9	80	5/13	4
Cefquinome	$[M+H]^+$	4.30	1	529.1	134.0[a]/166.9	140	13/25	4
Cefquinome-d_7	$[M+H]^+$	4.29	1	536.2	141.1	110	13	4
Mecillinam	$[M+H]^+$	4.41	1	326.2	167.0[a]/139.0	170	25/37	4
Cefalonium	$[M+H]^+$	4.42	1	459.1	151.9[a]/337.0	110	17/5	5
Cefotaxime	$[M+H]^+$	4.48	1	456.1	396.0[a]/125.0	140	9/50	4
Cefazolin	$[M+H]^+$	4.67	1	455.0	323.0[a]/155.9	110	9/13	4
Cefazolin-$^{13}C_2{}^{15}N$	$[M+H]^+$	4.67	1	458.1	326.0	110	5	4
Cefuroxime	$[M+NH_4]^+$	4.85	1	442.1	364.0[a]/336.1	110	5/13	4
Faropenem	$[M+NH_4]^+$	4.99	1	303.1	200.0[a]/182.0	80	5/21	4
Cefoperazone	$[M+H]^+$	5.16	1	646.2	143.0[a]/530.0	170	41/9	4
Ticarcillin	$[M+H]^+$	5.18	1	385.1	159.9[a]/114.0	140	9/45	4
DCD	$[M+2H]^{2+}$	5.26	1	429.0	182.9[a]/397.0	110	21/9	5
Ceftiofur	$[M+H]^+$	5.68	1	524.0	241.0[a]/125.3	170	17/50	4
Methicillin	$[M+H]^+$	5.77	1	381.1	164.9[a]/222.0	140	21/17	4
Piperacillin	$[M+H]^+$	5.82	1	518.2	143.0[a]/159.9	170	21/5	4
Piperacillin-d_5	$[M+H]^+$	5.81	1	523.2	148.0	170	17	5
Penicillin G	$[M+H]^+$	5.98	1	335.1	176.0[a]/160.0	110	13/5	4
Penicillin G-d_7	$[M+H]^+$	5.95	1	342.2	183.0	110	13	4
Penicillin V	$[M+H]^+$	6.31	1	351.1	159.9[a]/114.0	140	5/37	4
Penicillin V-d_5	$[M+H]^+$	6.29	1	356.1	160.0	140	5	4
Oxacillin	$[M+H]^+$	6.56	1	402.1	243.0[a]/114.0	110	9/49	4
Cloxacillin	$[M+H]^+$	6.85	1	436.1	159.9[a]/276.9	110	9/13	4
Nafcillin	$[M+H]^+$	6.93	1	415.1	199.0[a]/170.9	110	9/45	4
Nafcillin-d_5	$[M+H]^+$	6.92	1	420.2	204.1	110	9	4
Dicloxacillin	$[M+H]^+$	7.22	1	470.0	159.9[a]/310.9	140	9/13	4

[a] = Quantifier; RT = Retention Time; FRM = Fragmentor; CE = Collision Energy; CAV = Cell Accelerator Voltage; DAC = Desacetyl cephapirin; DCCD = Desfuroylceftiofur cysteine disulfide; DCD = Desfuroylceftiofur dimer.

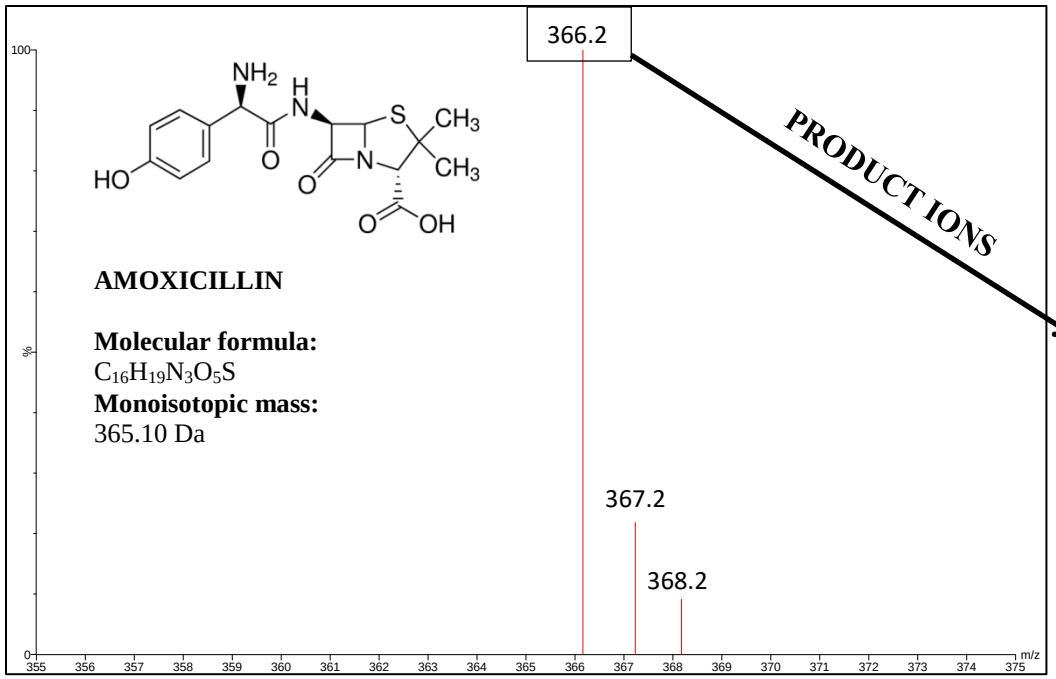
Table 3

Analyte	S/N[a] (Level, µg kg^{-1})	Estimated LOD (µg kg^{-1})	Estimated LOQ (µg kg^{-1})	ME%[b]	RSD(%) (without IS)	RSD(%) (with IS)
Amoxicillin	21(1)	0.15	0.50	28.7	8.2	4.8
Ampicillin	43(1)	0.075	0.25	-14.5	8.4	4.9
Cloxacillin	303(7.5)	0.075	0.25	-27.7	16.6	12.9
Dicloxacillin	107(7.5)	0.20	0.70	-40.4	22.9	13.0
Mecillinam	167(0.5)	0.0090	0.030	3.6	7.4	4.5
Methicillin	77(0.5)	0.020	0.065	-54	22.3	-
Nafcillin	1154(7.5)	0.020	0.065	-34.9	29.1	3.2
Oxacillin	152(7.5)	0.15	0.50	-9.3	14.5	-
Penicillin G	154(1)	0.020	0.065	-60	16.4	5.2
Penicillin V	334(1)	0.0090	0.030	-8.9	7.2	4.1
Piperacillin	21(1)	0.15	0.50	-35.9	10.1	3.1
Ticarcillin	52(2.5)	0.15	0.50	-3.6	16.2	-
Cefacetrile	625(31.25)	0.15	0.50	12.2	8.2	6.8
Cefadroxil	101(5)	0.15	0.50	29.4	5.9	2.3
Cephalexin	502(25)	0.15	0.50	-12.5	6.8	3.9
Cephapirin	1875(15)	0.025	0.080	-2.6	12.4	-
Cefalonium	333(5)	0.045	0.15	-31.3	16.6	13.4
Cefazolin	417(12.5)	0.090	0.30	-25.0	26.0	3.7
Cefoperazone	251(12.5)	0.15	0.50	-46.0	15.5	-
Cefotaxime	82(2)	0.065	0.25	-19.7	22.4	16.0
Cefquinome	202(5)	0.065	0.25	-13.4	9.5	3.0
Ceftiofur	278(25)	0.30	0.90	-55	28.2	-
Cefuroxime	101(5)	0.15	0.50	-59	18.4	16.4
DAC	302(15)	0.15	0.50	-3.1	10.8	2.7
DCCD	102(25)	0.65	2.5	-35.1	9.2	2.6
DCD	52(25)	1.5	5.0	15.3	27.3	-
Biapenem	31(1.5)	0.15	0.50	5.8	11.0	-
Doripenem	52(10)	0.60	2.0	8.3	8.7	6.9
Ertapenem	26(5)	0.60	2.0	-0.25	6.8	5.8
Imipenem	103(5)	0.15	0.50	-33.2	17.0	-
Meropenem	42(2.5)	0.20	0.60	-52	9.2	3.2
Faropenem	104(1)	0.030	0.10	-65	39.0	38.9

IS = Internal Standard; ME = Matrix Effect; [a] = Signal-to-noise ratio for the qualifier ion; [b] = Results are based on the analysis of 25 different bovine milk samples.

Table 4

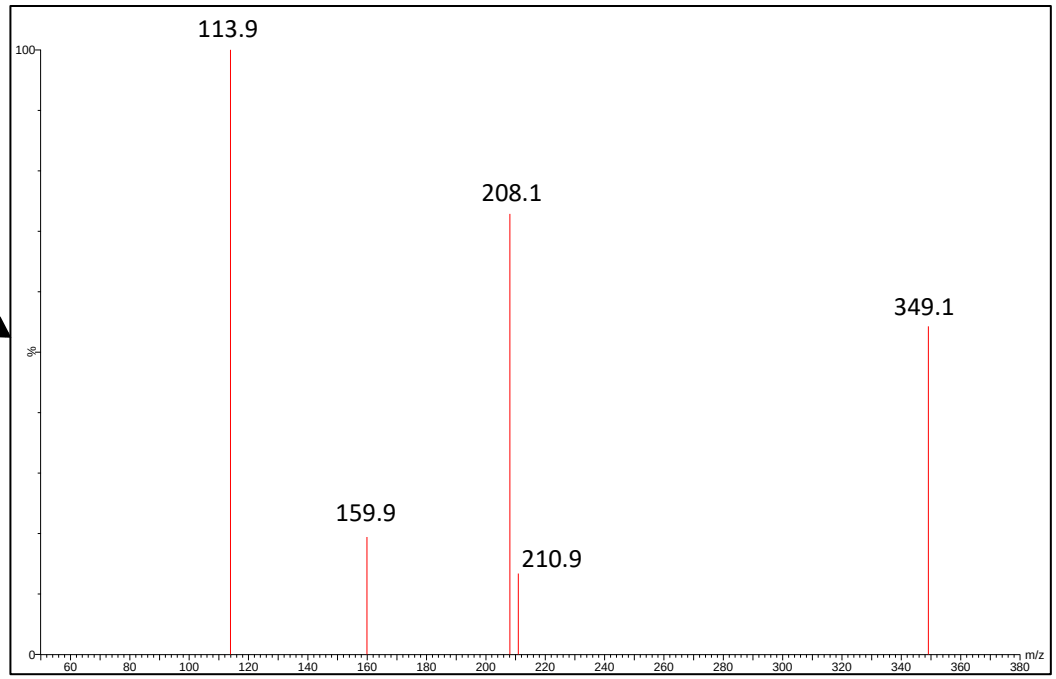
Analyte	Trueness% (WLr%)			Trueness% (WLR%)			CCα (µg kg^{-1})	CCβ (µg kg^{-1})
	0.5 MRL/TL	MRL/TL	1.5 MRL/TL	0.5 MRL/TL	MRL/TL	1.5 MRL/TL		
Amoxicillin	102 (3.6)	100 (3.4)	100 (3.1)	100 (5.2)	101 (4.8)	99 (2.0)	4.3	4.5
Ampicillin	103 (3.1)	100 (3.1)	101 (2.0)	99 (4.5)	98 (5.0)	97 (3.5)	4.2	4.5
Cloxacillin	99 (8.7)	93 (8.5)	91 (5.2)	100 (8.4)	96 (14.2)	94 (10.8)	38.7	49.0
Dicloxacillin	101 (8.6)	101 (7.9)	100 (5.4)	102 (10.1)	101 (12.4)	100 (6.0)	34.9	39.8
Mecillinam	101 (3.7)	100 (3.4)	101 (2.8)	103 (10.1)	95 (10.1)	101 (7.0)	2.1	2.4
Methicillin	106 (6.7)	108 (6.2)	107 (4.3)	91 (14.8)	96 (15.3)	96 (12.4)	2.4	3.0
Nafcillin	102 (1.8)	101 (1.9)	100 (1.1)	101 (1.9)	101 (1.5)	100 (1.4)	30.8	31.6
Oxacillin	103 (8.2)	109 (6.6)	115 (5.3)	96 (15.8)	98 (16.0)	100 (9.5)	39.0	47.0
Penicillin G	100 (3.3)	99 (1.9)	97 (2.5)	99 (3.6)	99 (4.0)	99 (2.6)	4.1	4.4
Penicillin V	101 (3.3)	100 (2.8)	100 (2.1)	103 (4.2)	101 (2.3)	101 (3.3)	4.1	4.3
Piperacillin	99 (3.8)	99 (2.5)	99 (1.8)	101 (3.1)	100 (2.5)	99 (1.7)	4.1	4.3
Ticarcillin	102 (2.9)	102 (4.3)	102 (5.6)	110 (38.6)	105 (29.9)	119 (36.0)	11.8	25.1
Cefacetrile	97 (4.9)	95 (2.7)	96 (2.5)	97 (6.7)	97 (5.3)	98 (4.9)	133	145
Cefadroxil	98 (2.9)	100 (2.6)	99 (2.8)	99 (2.8)	100 (3.0)	98 (3.0)	21.0	22.0
Cephalexin	101 (0.86)	100 (1.1)	100 (1.0)	100 (2.8)	100 (3.2)	100 (1.6)	104	108
Cephapirin	106 (4.5)	105 (3.3)	102 (2.7)	103 (18.9)	101 (18.4)	112 (15.8)	69	90
Cefalonium	103 (3.5)	102 (2.6)	101 (2.1)	103 (18.3)	109 (15.3)	106 (10.7)	22.2	27.2
Cefazolin	100 (1.2)	100 (1.1)	100 (1.2)	100 (2.2)	100 (1.4)	100 (1.9)	51	52
Cefoperazone	100 (6.2)	105 (4.5)	105 (3.7)	105 (34.7)	107 (29.3)	121 (35.1)	60	124
Cefotaxime	101 (3.7)	99 (3.3)	97 (3.1)	103 (17.0)	104 (25.5)	104 (16.5)	9.7	13.2
Cefquinome	101 (1.8)	101 (1.1)	101 (1.5)	101 (2.6)	103 (5.0)	102 (4.2)	21.0	22.5
Ceftiofur	102 (8.3)	108 (7.7)	110 (5.3)	94 (18.4)	101 (15.0)	102 (9.6)	126	151
Cefuroxime	100 (4.9)	95 (4.2)	93 (3.3)	104 (18.9)	108 (21.8)	108 (18.3)	24.9	34.6
DAC	100 (1.5)	100 (1.4)	100 (1.3)	99 (2.9)	99 (3.4)	100 (2.0)	62	64
DCCD	101 (2.5)	100 (1.3)	100 (1.5)	101 (2.3)	100 (1.6)	100 (1.7)	102	105
DCD	104 (17.7)	107 (9.7)	111 (6.4)	107 (25.3)	109 (29.4)	130 (20.8)	124	182
Biapenem	105 (4.3)	104 (3.5)	103 (3.8)	106 (12.6)	110 (10.2)	110 (11.7)	7.6	9.3
Doripenem	102 (3.8)	98 (3.3)	98 (2.6)	98 (5.7)	102 (5.4)	102 (2.9)	42.6	45.3
Ertapenem	97 (4.3)	98 (4.7)	95 (5.7)	98 (9.0)	96 (9.1)	98 (6.4)	21.3	23.9
Imipenem	100 (4.9)	101 (3.1)	93 (8.7)	99 (21.1)	100 (10.2)	99 (10.6)	23.7	28.3
Meropenem	101 (2.8)	101 (3.3)	100 (2.1)	98 (4.0)	98 (3.7)	99 (2.1)	10.5	11.0
Faropenem	99 (5.8)	98 (4.1)	97 (5.2)	97 (24.6)	106 (25.0)	106 (20.4)	7.3	11.4

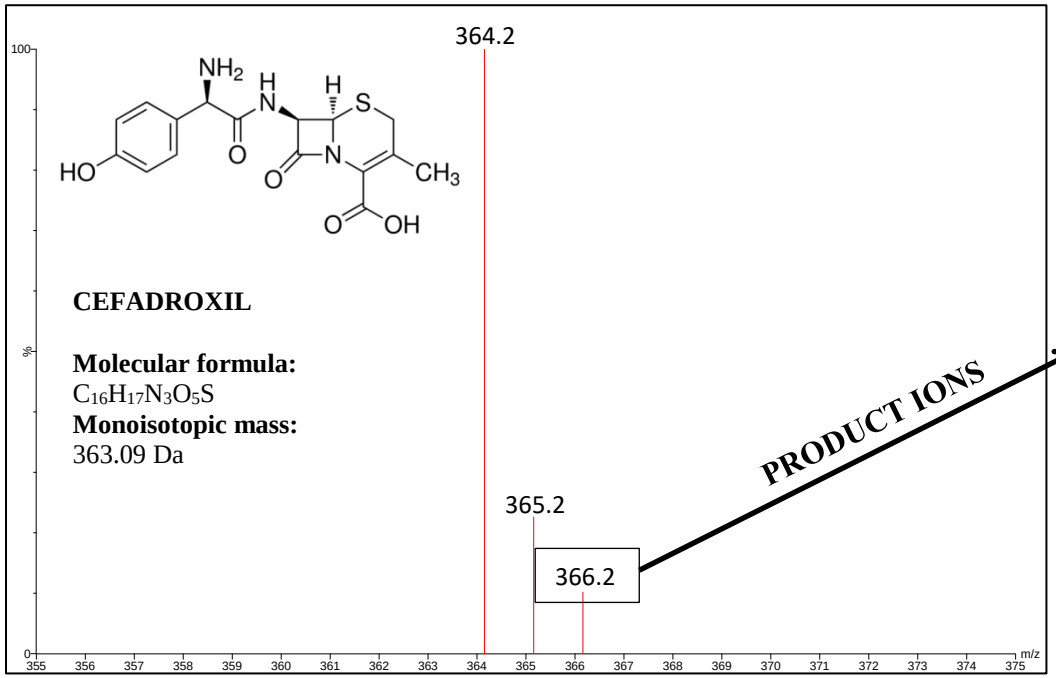


AMOXICILLIN

Molecular formula:
$C_{16}H_{19}N_3O_5S$
Monoisotopic mass:
365.10 Da

PRODUCT IONS

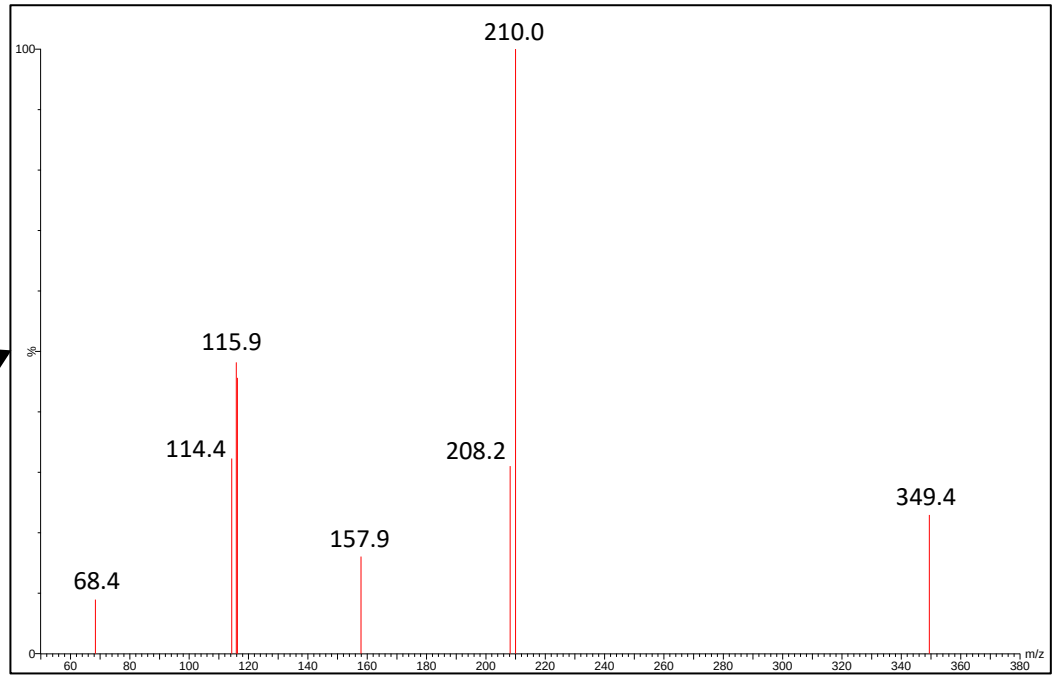


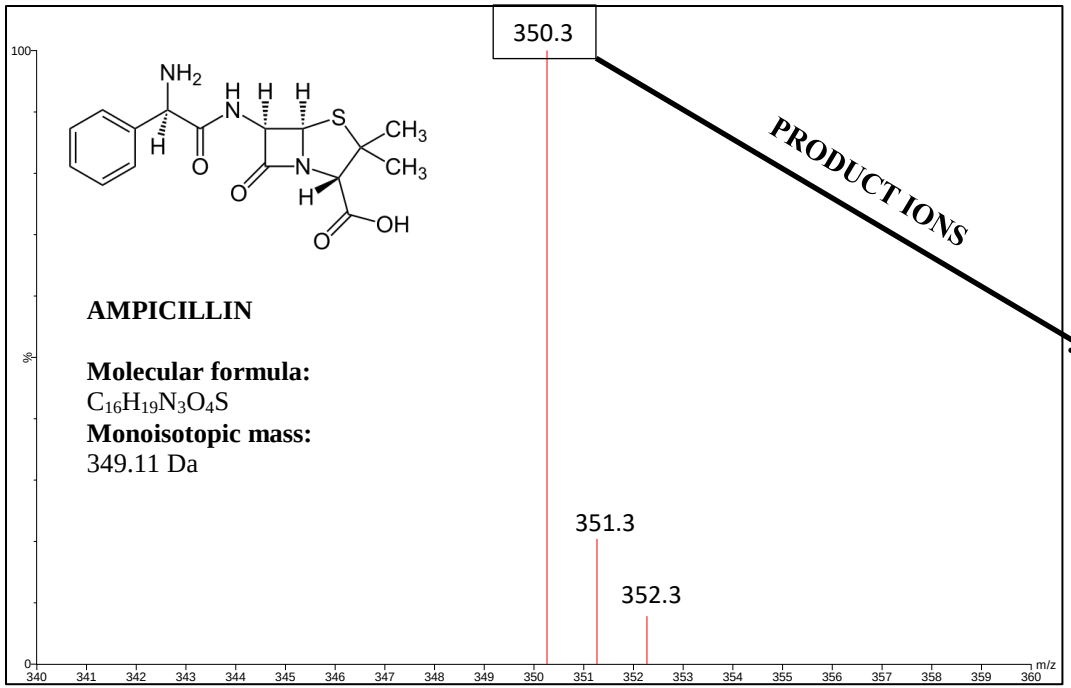


CEFADROXIL

Molecular formula:
$C_{16}H_{17}N_3O_5S$
Monoisotopic mass:
363.09 Da

PRODUCT IONS

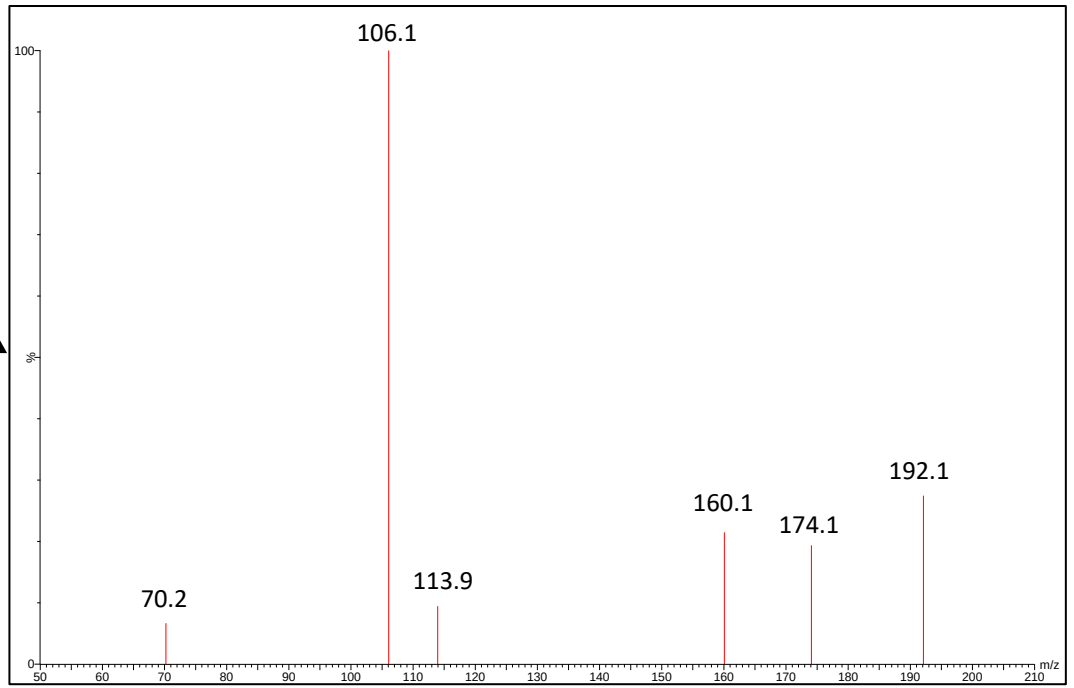


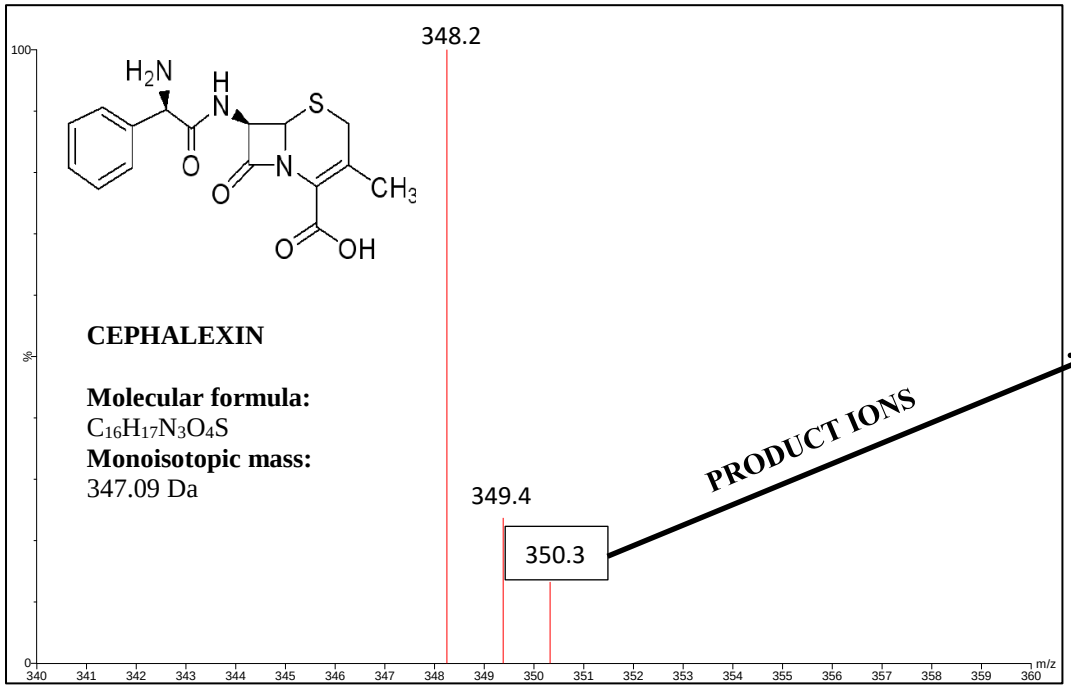


AMPICILLIN

Molecular formula:
$C_{16}H_{19}N_3O_4S$
Monoisotopic mass:
349.11 Da

PRODUCT IONS

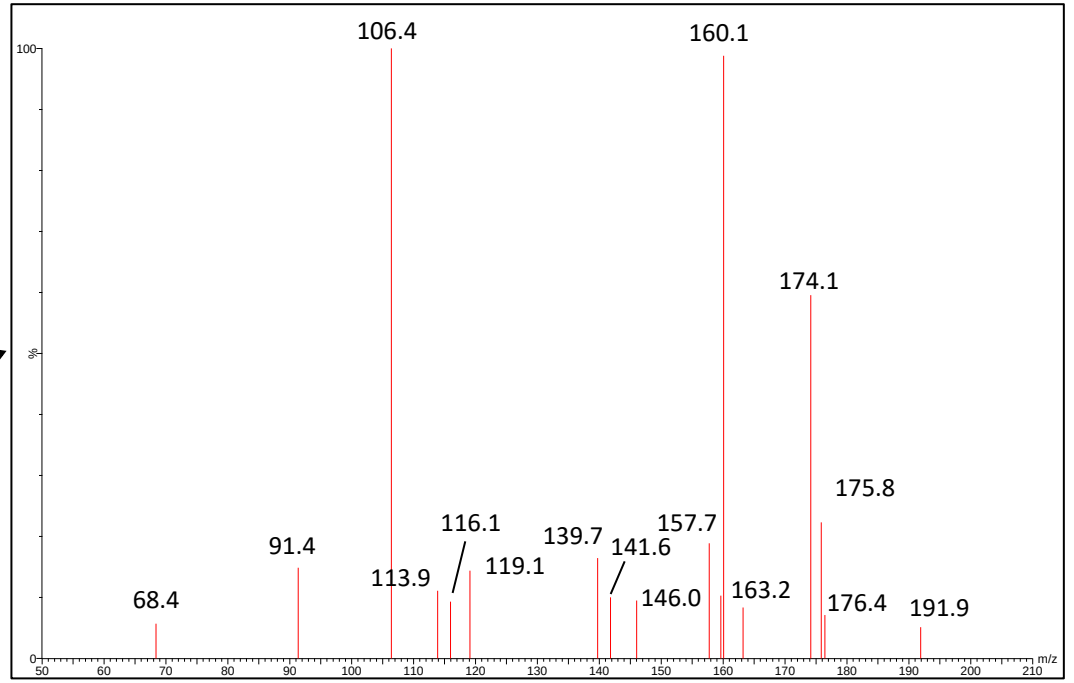




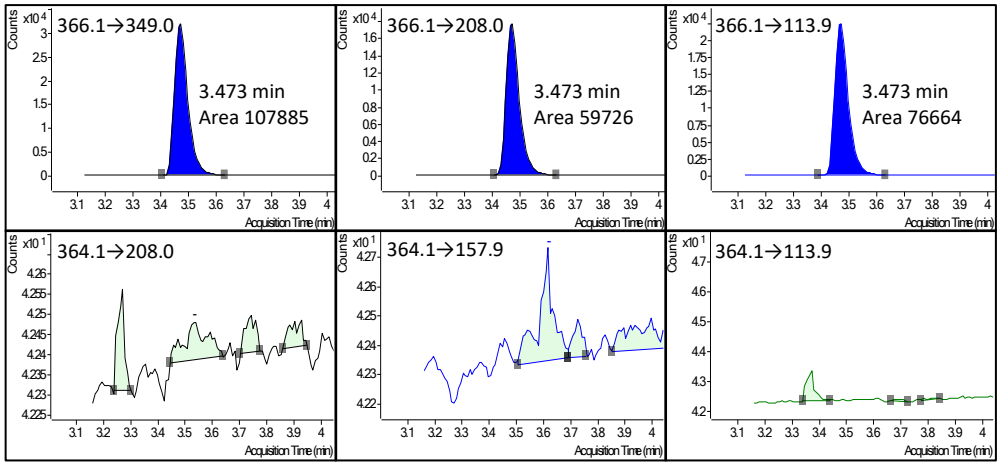
CEPHALEXIN

Molecular formula:
$C_{16}H_{17}N_3O_4S$
Monoisotopic mass:
347.09 Da

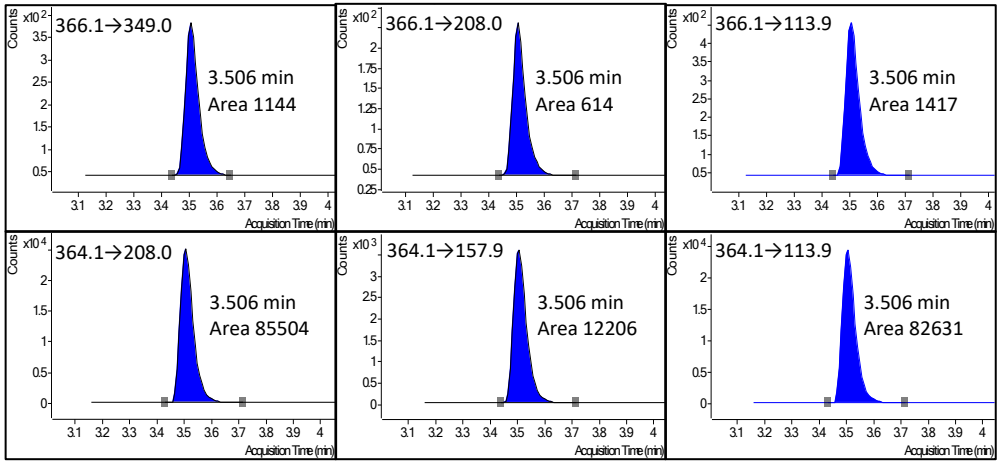
PRODUCT IONS



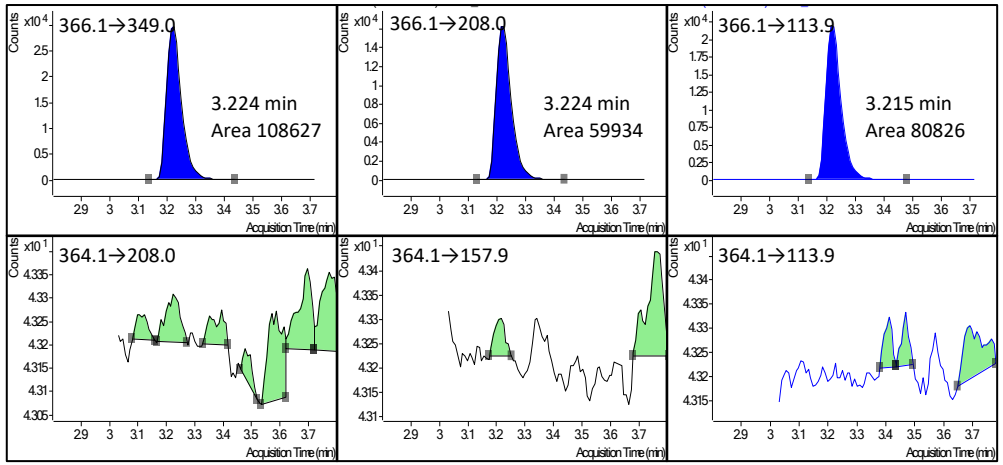
A: Injection of amoxicillin standard (CSH C_{18})



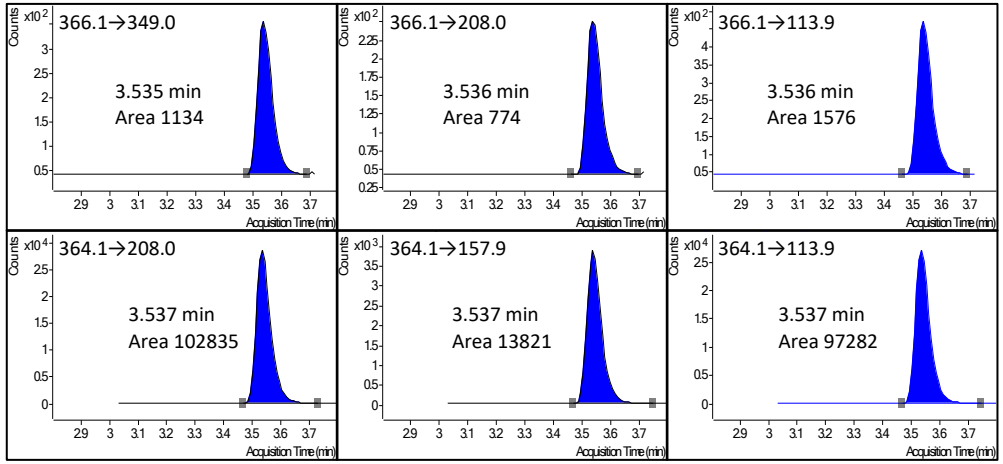
B: Injection of cefadroxil standard (CSH C_{18})



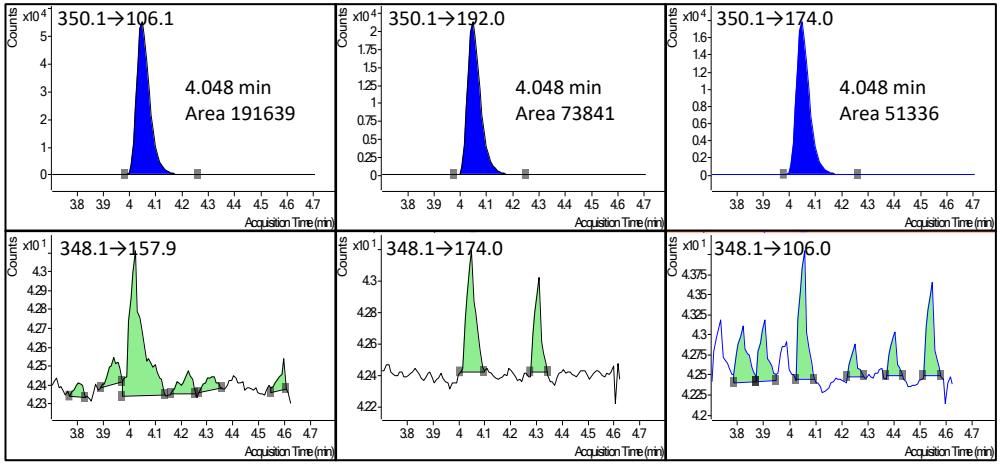
C: Injection of amoxicillin standard (phenyl-hexyl)



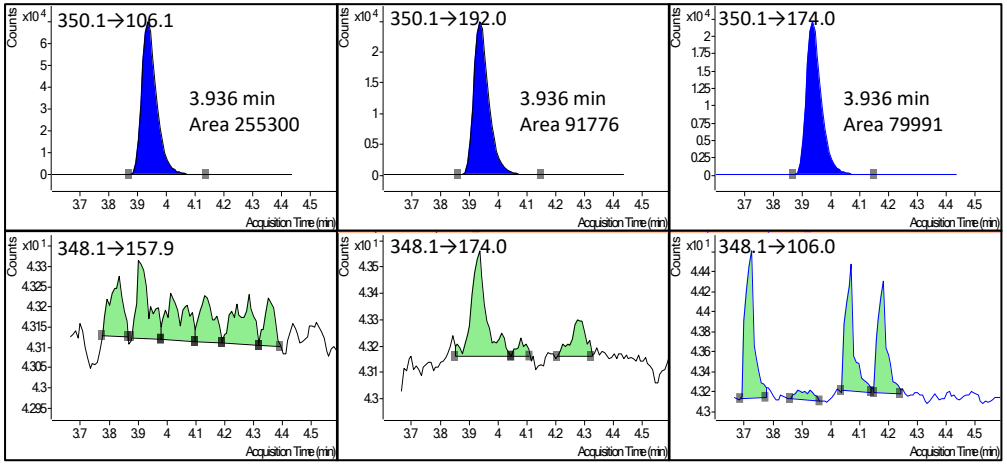
D: Injection of cefadroxil standard (phenyl-hexyl)



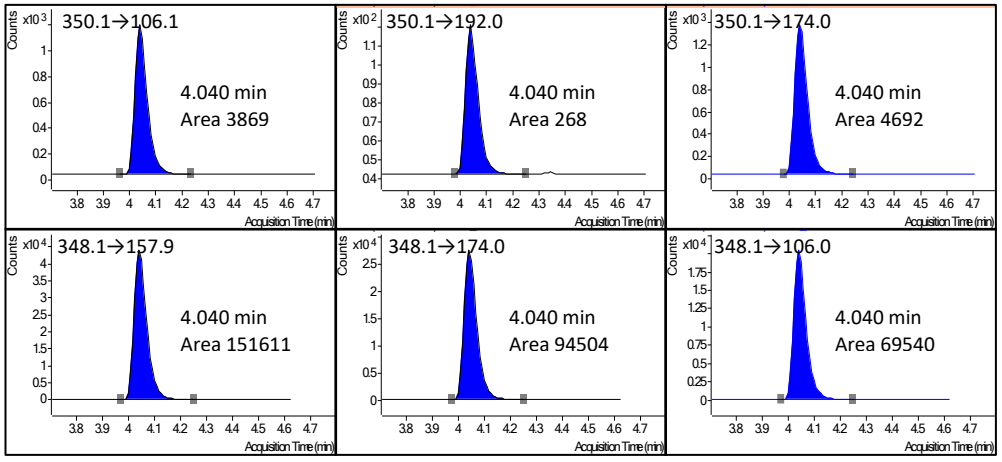
A: Injection of ampicillin standard (CSH C_{18})



C: Injection of ampicillin standard (phenyl-hexyl)



B: Injection of cephalexin standard (CSH C_{18})



D: Injection of cephalexin standard (phenyl-hexyl)

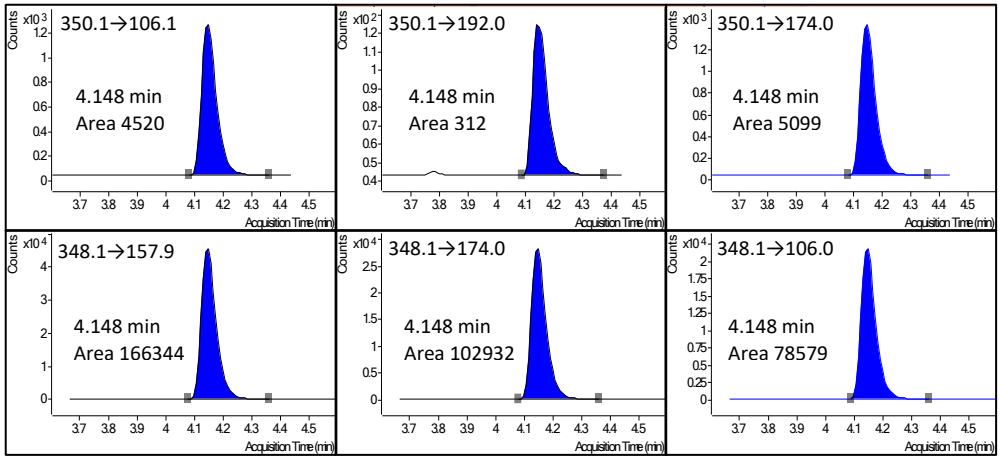
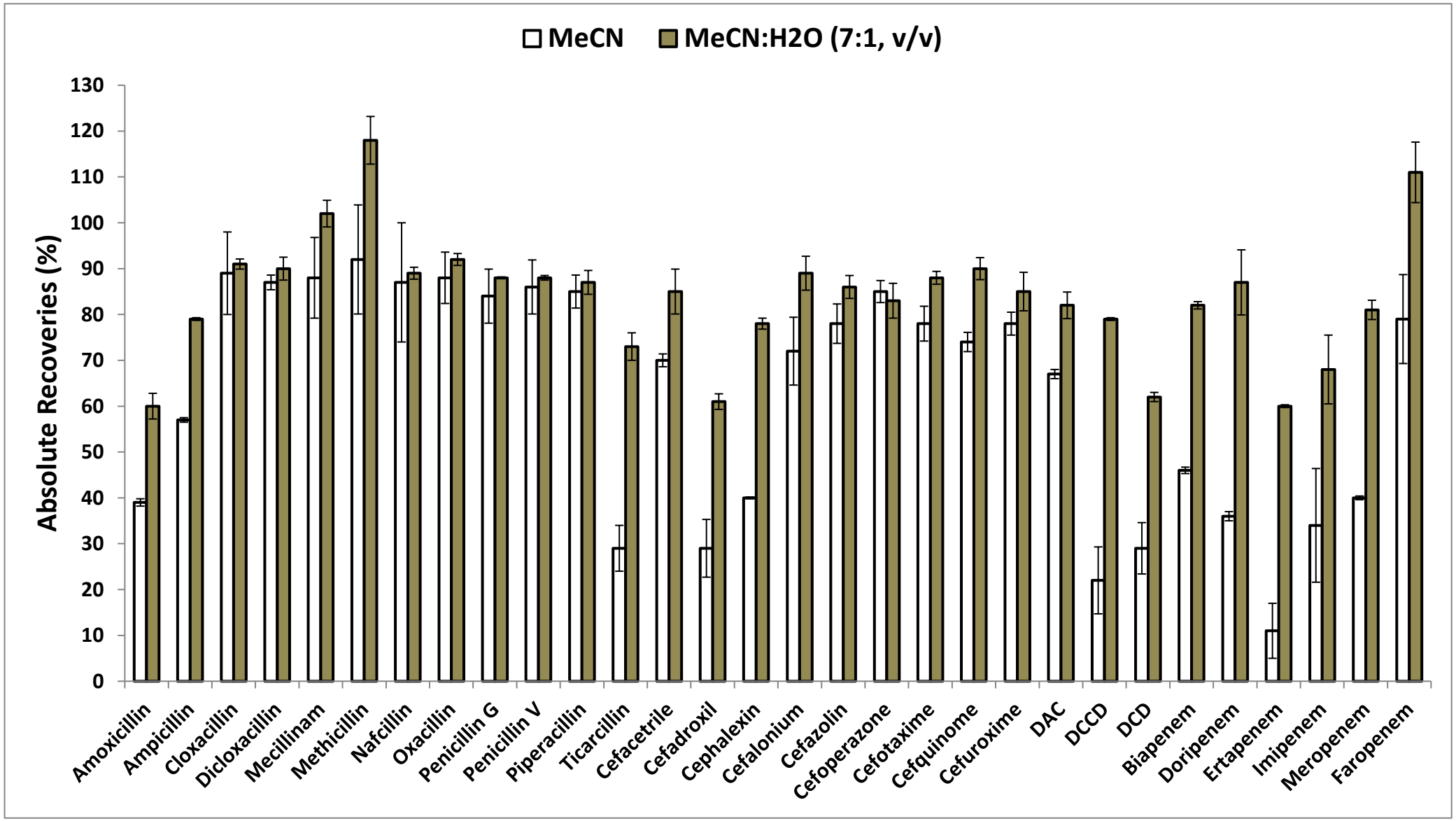
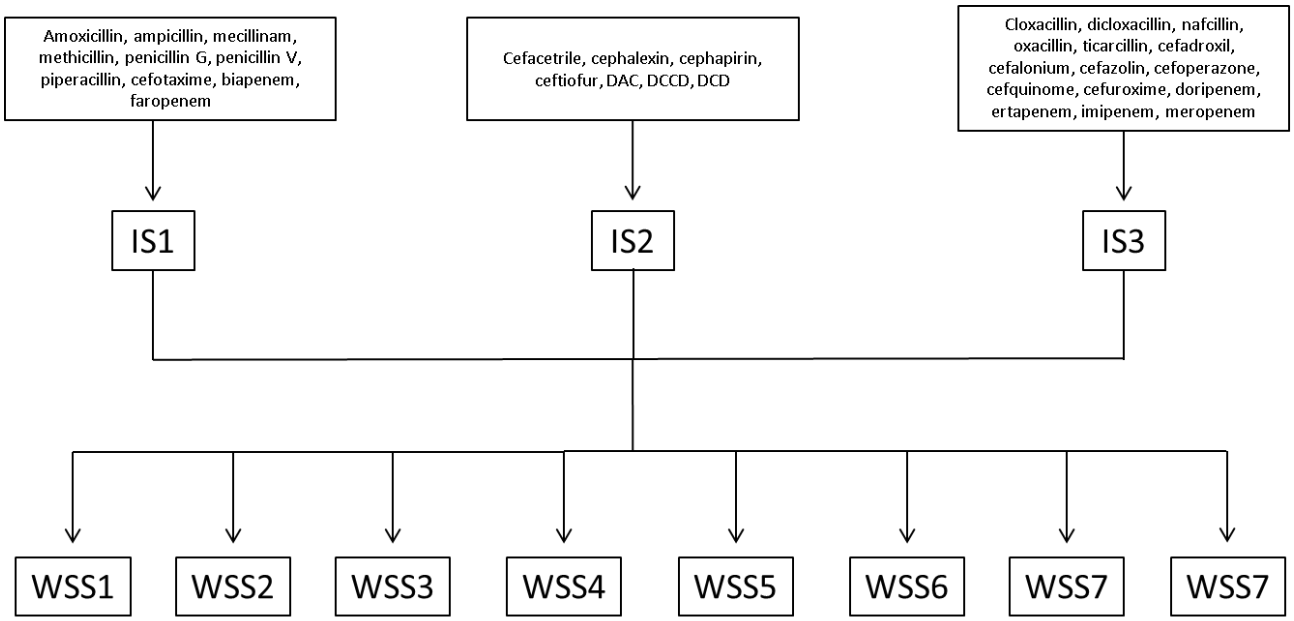


Fig3


MeCN
MeCN:H2O (7:1, v/v)
Absolute Recoveries (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Amoxicillin
Ampicillin
Cloxacillin
Dicloxacillin
Mecillinam
Methicillin
Nafcillin
Oxacillin
Penicillin G
Penicillin V
Piperacillin
Ticarcillin
Cefacetrile
Cefadroxil
Cephalexin
Cefalonium
Cefazolin
Cefoperazone
Cefotaxime
Cefquinome
Cefuroxime
DAC
DCCD
DCD
Biapenem
Doripenem
Ertapenem
Imipenem
Meropenem
Faropenem

Online Resource 1

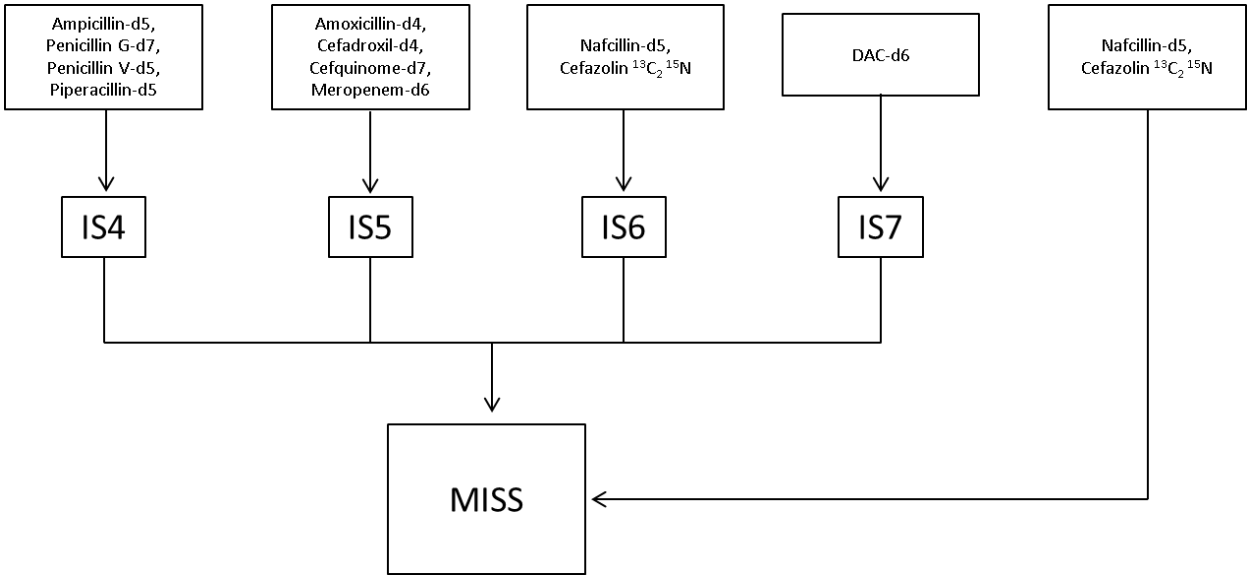


Online Resource 2

Analyte	IS1 (µg mL^{-1})	IS2 (µg mL^{-1})	IS3 (µg mL^{-1})	Range WSS1-WSS8 (µg mL^{-1})
Amoxicillin	8	-	-	0.02-0.16
Ampicillin	8	-	-	0.02-0.16
Cloxacillin	-	-	12	0.15-1.2
Dicloxacillin	-	-	12	0.15-1.2
Mecillinam	4	-	-	0.01-0.08
Methicillin	4	-	-	0.01-0.08
Nafcillin	-	-	12	0.15-1.2
Oxacillin	-	-	12	0.15-1.2
Penicillin G	8	-	-	0.02-0.16
Penicillin V	8	-	-	0.02-0.16
Piperacillin	8	-	-	0.02-0.16
Ticarcillin	-	-	4	0.05-0.4
Cefacetrile	-	50	-	0.625-5
Cefadroxil	-	-	8	0.1-0.8
Cephalexin	-	40	-	0.5-4
Cephapirin	-	24	-	0.3-2.4
Cefalonium	-	-	8	0.1-0.8
Cefazolin	-	-	20	0.25-2
Cefoperazone	-	-	20	0.25-2
Cefotaxime	16	-	-	0.04-0.32
Cefquinome	-	-	8	0.1-0.8
Ceftiofur	-	40	-	0.5-4
Cefuroxime	-	-	8	0.1-0.8
DAC	-	24	-	0.3-2.4
DCCD	-	40	-	0.5-4
DCD	-	40	-	0.5-4
Biapenem	12	-	-	0.03-0.24
Doripenem	-	-	16	0.2-1.6
Ertapenem	-	-	8	0.1-0.8
Imipenem	-	-	8	0.1-0.8
Meropenem	-	-	4	0.05-0.4
Faropenem	8	-	-	0.02-0.16

IS = Intermediate Solution; WSS = Working Standard Solution.

Online Resource 3



Online Resource 4

Internal standard	IS4 (µg mL^{-1})	IS5 (µg mL^{-1})	IS6 (µg mL^{-1})	IS7 (µg mL^{-1})	Final MISS (µg mL^{-1})
Amoxicillin-d4	-	20	-	-	0.16
Ampicillin-d5	10	-	-	-	0.08
Nafcillin-d5	-	-	37.5	-	0.6
Penicillin G-d7	10	-	-	-	0.08
Penicillin V-d5	10	-	-	-	0.08
Piperacillin-d5	10	-	-	-	0.08
Cefadroxil-d4	-	50	-	-	0.4
Cefalexin-d5	-	-	-	-	2
Cefazolin $^{13}C_2$, ^{15}N	-	-	62.5	-	1
Cefquinome-d7	-	50	-	-	0.4
DAC-d6	-	-	-	37.5	1.2
DCCD-d3	-	-	-	-	2
Meropenem-d6	-	25	-	-	0.2

IS = Intermediate Solution; MISS = Mixed Internal Standard Solution.